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Idiotypic Cascades after Injection of the Monoclonal Antibody OC125: A Study in a Mouse Model

Induction of Antibodies against OC125 and CA 125 after Immunization with an Anti-CA 125 (MAb OC125) Monoclonal Antibody by Activation of the Idiotypic Network

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Summary: By immunization of mice with the anti-CA 125 monoclonal antibody OC125, we tried to induce antibodies directed against the tumour-associated antigen CA 125, via activation of the idiotypic network. Mice immunized by repeated administrations of $F(ab')_2$-fragments of the OC125 antibody produced anti-idiotypic antibodies, imitating the original target antigen of the OC125. After induction of these anti-idiotypic antibodies (Ab2β) a murine IgG-anti-CA 125 (Ab3) response was detected. The induction of idiotypic cascades offers the possibility of immunization against tumour-associated antigens, without using the original antigen and breaking antitumour tolerance.

Introduction

Niels Jerne postulated that the immune system consists of a network of antibodies and lymphocytes which interact through complementary structures of antibodies, anti-antibodies or anti-anti-antibodies (1). It is thus proposed that an antigen causes the production of antibodies (Ab1), bearing different idiotopes characteristic for each antibody clone. These idiotopes can act as antigens and stimulate the production of anti-idiotypic antibodies (Ab2), which control the production of Ab1, and which in turn are controlled by anti-anti-idiotypic antibodies (Ab3).

Within the set of anti-idiotypic antibodies directed against the several idiotopes of Ab1, some bind exactly to the antigen binding site. According to the classification originally proposed by *Jerne* (2) and later modified by *Bona & Köhler* (3), these anti-idiotypic antibodies are designated as Ab2β or so called internal image anti-idiotype. The idiotope of an Ab2β type anti-idiotypic antibody has the shape of the original antigenic determinant and acts like an internal image of the antigen. The other anti-idiotypic antibodies are called Ab2α and Ab2γ. The Ab2α recognizes a non-antigen combining site idiotope somewhere on the hypervariable region; the Ab2γ recognizes an idiotope in close proximity to the antigen combining site but is not an internal image and may be representative of recurrent intrastrain or intraspecies idiotopes. The Ab2γ, like the Ab2β, recognizes an idiotope within the antigen binding site, but fails to exhibit biological mimicry of the antigen. The Ab2β can induce the formation of anti-anti-idiotypic antibodies (Ab3), which can again bind to the primary antigen in so far as they fit into the binding site of the Ab2β (1, 4–8).

We previously demonstrated the appearance of anti-idiotypic antibodies mimicking the tumour-associated antigen CA 125, in ovarian cancer patients after re-

peated treatment with $F(ab')_2$-fragments of the anti-CA 125 antibody OC125 (9). Induction of these anti-idiotypic antibodies obviously leads to an improved survival rate, even in advanced stages (10, 11), by presumably breaking antitumour tolerance. A common explanation for the absence of antitumour immunity is that the immune system has been tolerized by the tumour antigen (12). An effective method of breaking this tolerance is to present the critical epitope in a different molecular environment to the tolerized host. According to the network hypothesis of *Jerne* (1), one approach is the transformation of epitope structures into idiotypic determinants expressed on the surface of antibodies.

The aim of this study was to evaluate in an animal model the induction of anti-idiotypic antibodies (Ab2β), which mimick the tumour-associated antigen CA 125, i. e. to determine whether immunization with $F(ab')_2$-fragments of OC125 antibody subsequently leads to the production of anti-anti-antibodies (Ab3) directed against the target antigen CA 125.

Materials and Methods

Materials

The $F(ab')_2$-fragments of the monoclonal antibody OC125 MAb, which bind to the tumour-associated-antigen CA 125, were generously donated from International CIS, Filiale de Compagnie ORIS Industrie S. A. (Gis-sur-Yvette, France).

The human ovarian adenocarcinoma cell line OAW42 was supplied by *H. Löhrke*, Deutsches Krebsforschungszentrum (Heidelberg, Germany) and cultured in RPMI medium (Biochrom KG, Berlin, Germany), supplemented with fetal-calf serum (Biochrom KG, Berlin, Germany), volume fraction 0.1, 2 mmol/l *L*-glutamine (Boehringer-Mannheim, Mannheim, Germany) and streptomycin/penicillin (Biochrom KG, Berlin, Germany).

Ninety six-well flat bottom Dynatech microtitre plates were obtained from Greiner GmbH & CoKG, Nürtingen, Germany. Globulin-free bovine serum albumin, peroxidase-labelled goat anti-mouse-IgG (F_c-specific) and peroxidase-labelled goat anti-mouse-IgM were purchased from Sigma Chemie GmbH (Deisenhofen, Germany) and used without further purification. The peroxidase substrate, *o*-phenylenediamine, was obtained from Zymed Laboratories Inc., San Francisco, USA. All other chemicals and solvents were obtained from Merck (Darmstadt, Germany) or Aldrich (Steinheim, Germany).

Buffers

Buffer A:
0.1 mol/l sodium phosphate, pH 7.4; 0.15 mol/l NaCl

Buffer B:
0.5 mol/l sodium citrate-phosphate pH 5.5; 3 ml/l H_2O_2

Buffer C (Washing buffer):
0.1 mol/l sodium phosphate, pH 7.4; 0.15 mol/l NaCl, 1 ml/l Tween 20

Buffer D (Blocking buffer):
0.1 mol/l sodium phosphate, pH 7.4; 0.15 mol/l NaCl, 10 g/l bovine serum albumin

Buffer E (Incubation buffer):
0.1 mol/l sodium phosphate, pH 7.4; 0.15 mol/l NaCl, 5 g/l bovine serum albumin

Immunization protocol

For immunization, $F(ab)_2$-fragments of the monoclonal antibody OC125 MAb were coupled to maleimide-activated key-hole-limpet-haemocyanin (Pierce Chemical Co., Rockford, USA) according to the product information. Female BALB/c mice, 9 to 10 week old, received intraperitoneally 100 µg $F(ab)_2$-fragments in incomplete *Freund*'s Adjuvant (Behring, Marburg, Germany) on day 1, 31 and 61. The animals received a final boost with 50 µg unconjugated $F(ab)_2$-fragments of the monoclonal antibody OC125 MAb in buffer A on day 75. Blood samples were collected on day 0 (control), 8, 38, 68 and 79.

Measurement of CA 125

CA 125 concentration was determined by the CA 125 EIA (Abbott Diagnostik, Wiesbaden-Delkenheim, Germany) according to the instructions of the manufacturer.

Purification of CA 125

Culture supernatants of OAW42 cells, containing the CA 125 antigen, were collected after one week of cell growth and stored at −25 °C until use.

Ammonium sulphate fractionation

Ammonium sulphate (208 g/l) was added to the culture supernatant. The mixture was centrifuged (10 000 g, 20 min) and the precipitate discarded. To the supernatant fraction, 163 g/l ammonium sulphate was added followed by a second centrifigation. The precipitate was resuspended in buffer A and dialysed overnight against twenty volumes of buffer A.

Gel filtration on Superose 6

0.5 ml of the CA 125-containing ammonium sulphate fraction (35−60% saturation) was applied to a Superose 6 column (1.5 × 30 cm, Pharmacia, Freiburg, Germany), equilibrated with buffer A. Fractions containing CA 125 (8−11 ml elution volume) were pooled and concentrated by Centricon-30 (Amicon, Beverly, USA). Aliquots of 1 ml (5000 × 10^3 units/l) were stored at −25 °C until use.

Immunometric assays

Microtitre plates were coated with $F(ab')_2$-fragments of the OC125 or with CA 125 overnight at 4 °C in 100 µl coating buffer A per well. The coated wells were then washed once with washing buffer C (300 µl/well) and blocked with 200 µl buffer D per well for 1 h at room temperature. After three washes with buffer C, the immobilized antibody or antigen was incubated for 18 h at 4 °C with 100 µl/well of the serum samples diluted in incubation buffer E. The plates were then washed three times with buffer C and bound murine immunoglobulins were detected by incubation with peroxidase-labelled goat anti-mouse IgG or IgM (diluted 1 : 2000 or 1 : 5000, with buffer E) for 2 h at 37 °C (100 µl/well). Following three washes, 100 µl of substrate solution (1 g/l *o*-phenylendiamine in buffer B) was added. After incubation for 30 min at room temperature in the dark, reaction was stopped by addition of 100 µl 0.5 mol/l H_2SO_4 and absorbance was measured at 495 and 650 nm.

1a) *Determination of anti-OC125 antibodies*

For determination of the concentration of anti-OC125 antibodies, 96-well Dynatech microtitre plates were coated with $F(ab')_2$-fragments of OC125 at a concentration of 2 mg/l. Sera of the immunized mice and the control samples were incubated in different concentrations (diluted 1/100, 1/200, 1/400 and 1/800) and tested for bound mouse-IgG and mouse-IgM.

1b) *Inhibition of anti-OC125 antibody binding*

To determine the portion of anti-OC125 antibodies bound to the paratope of the OC125, an inhibition assay was performed. Microtitre plates were coated as described above with 200 ng/well of $F(ab')_2$-fragments of OC125, then incubated with 50 µl/well of the serum obtained on day 68 (diluted 1/100), together with 50 µl purified CA 125 in rising concentrations (0.003—30000 × 10^3 units/l) overnight at 4 °C. Bound anti-OC125 antibodies were detected as described and the percentage of inhibition was calculated in relation to serum incubated with 50 µl buffer E.

2a) *Determination of anti-CA 125 antibodies*

For determination of the concentration of anti-CA 125 antibodies, microtitre plates were coated with 100 µl/well of a solution of purified CA 125 (500 × 10^3 units/l) at 4 °C overnight, followed by incubation with 100 µl/well of serum from the immunized mice, or with control samples (diluted 1/100, 1/200, 1/400, 1/800) overnight at 4 °C. After washing, the plates were tested for bound mouse-IgG.

2b) *Inhibition of anti-CA 125 antibody binding*

For further characterization of anti-CA 125 antibodies, an inhibition assay was performed. Microtitre plates were coated as described above with 50 units/well of purified CA 125, then incubated with 50 µl/well of the serum samples obtained on day 68, together with 50 µl purified CA 125 (concentration range: 10^4—10^8 units/l) overnight at 4 °C. Bound anti-CA 125 was detected as described and the percentage of inhibition was calculated in relation to sera incubated together with 50 µl buffer E.

Results

CA 125 purification

In the purification procedure, the specific activity was raised from 4.5 to 252.2 units/mg protein. By ammonium sulphate precipitation, large amounts of IgG and albumin were separated from fetal calf serum. Chromatography on Superose 6 resulted in additional separation of IgG, albumin and low molecular weight proteins. The preparation showed no cross-reactivity in immunoblots after isoelectric focussing.

Success of immunization

Before immunization with $F(ab')_2$-fragments of the OC125, no anti-OC125 or anti-CA 125 antibodies could be detected (control samples one day before immunization).

After treatment with OC125 $F(ab')_2$-fragments, increasing concentrations of anti-OC125 antibodies were detected in sera of all mice (fig. 1). Antibodies of the IgM type appeared immediately after the first immunization and reached a maximum after the second immunization. For antibodies of the IgG type, the increase was delayed, and a maximum occurred after the third boost (fig. 2).

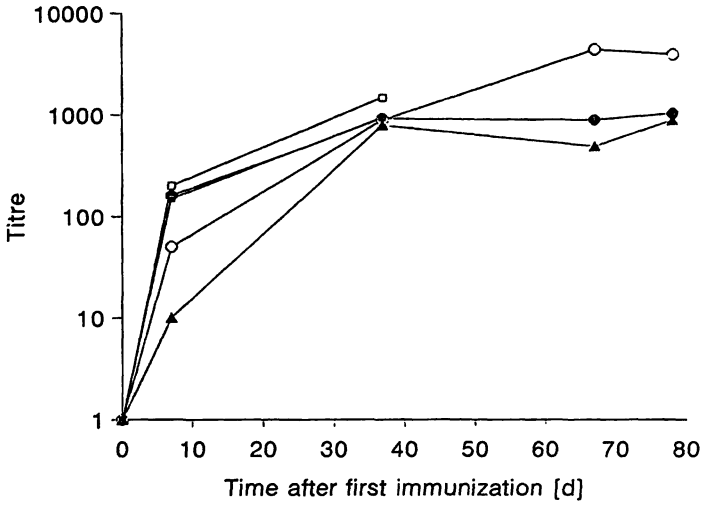

Fig. 1. The serum concentrations of anti-idiotypic IgG (Ab2β) during the immunization protocol of 5 mice. Anti-idiotypic antibodies were detected on $F(ab)_2$-OC125-coated microtitre plates by peroxidase-labelled anti-mouse IgG (F_c-specific) antibodies and *o*-phenylenediamine as substrate. The titre producing an absorbance of 0.050 at 495 nm is given (▲—▲ mouse 1, ▫—▫ mouse 2, ●—● mouse 3, ○—○ mouse 4, □—□ mouse 5).

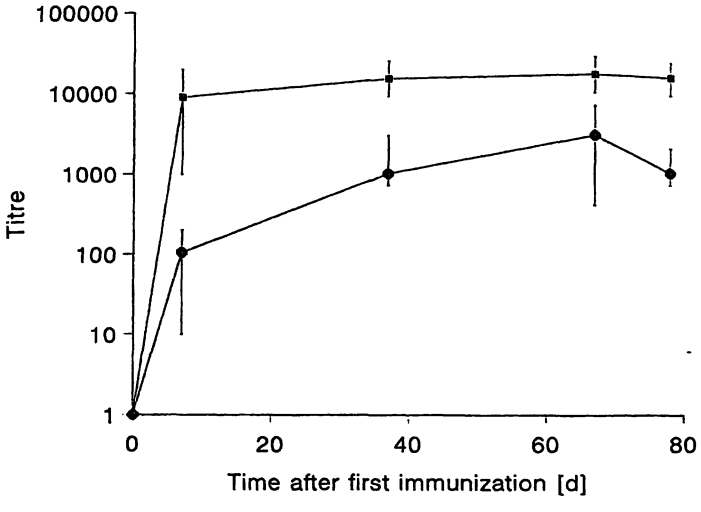

Fig. 2. Mean serum concentrations of anti-idiotypic antibodies in 5 mice. Anti-idiotypic IgG (●—●) and IgM (▪—▪) antibodies were determined during the immunization protocol on $F(ab)_2$-OC125-coated microtitre plates by peroxidase-labelled anti-mouse IgG (F_c-specific) antibodies or peroxidase-labelled anti-mouse IgM antibodies and *o*-phenylenediamine as substrate. The titre producing an absorbance of 0.050 at 495 nm is given. Serum samples were diluted 1/100 for IgG and 1/800 for IgM analysis.

The binding of anti-OC125 antibodies was inhibited by the nominal antigen CA 125 in a concentration dependent manner. Figure 3 demonstrates that the binding of anti-OC125 antibodies could be inhibited nearly completely by the addition of 30 000 × 10^3 units/l of CA 125. This indicates that most of the Ab2 recognize idiotopes at or near the binding site of the OC125.

We also found increasing concentrations of anti-CA 125 antibodies in sera of mice after immunization (fig. 4). Murine anti-CA 125 antibodies were detected even after the first immunization, and they reached a maximum after the second immunization. After the third and fourth boost, the anti-CA 125 titre was stable or slightly decreased.

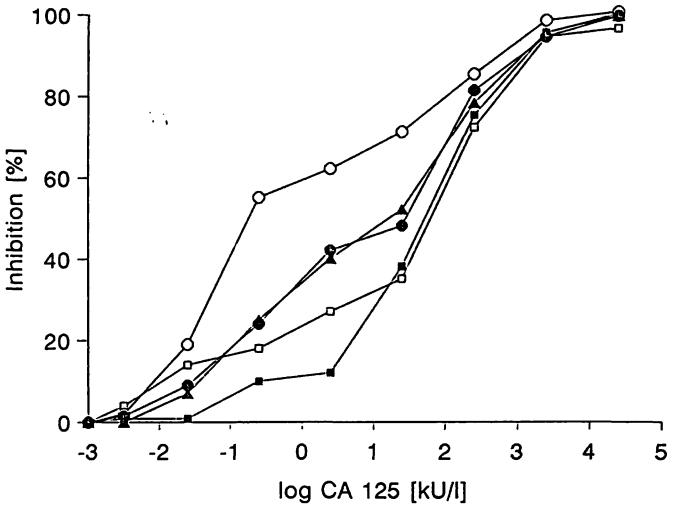

Fig. 3. Inhibition of the binding activity of anti-idiotypic IgG by increasing concentrations of CA 125 antigen (▲—▲ mouse 1, ■—■ mouse 2, ●—● mouse 3, ○—○ mouse 4, □—□ mouse 5).

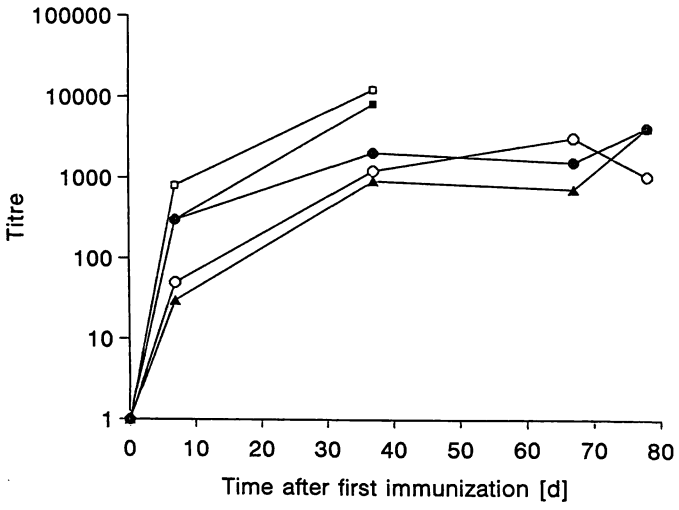

Fig. 4. The serum concentrations of anti-anti-idiotypic IgG (Ab3) during the immunization protocol of 5 mice. Anti-anti-idiotypic antibodies were detected on CA 125-coated microtitre plates by peroxidase-labelled anti-mouse IgG (F_c-specific) antibodies and *o*-phenylenediamine as substrate. The titre producing an absorbance of 0.050 at 495 nm is given ▲—▲ mouse 1, ■—■ mouse 2, ●—● mouse 3, ○—○ mouse 4, □—□ mouse 5).

The binding of these antibodies to immobilized CA 125 was inhibited by soluble CA 125 in a concentration dependent manner and the inhibition was nearly complete at 100 000 × 10^3 units/l of CA 125 (fig. 5).

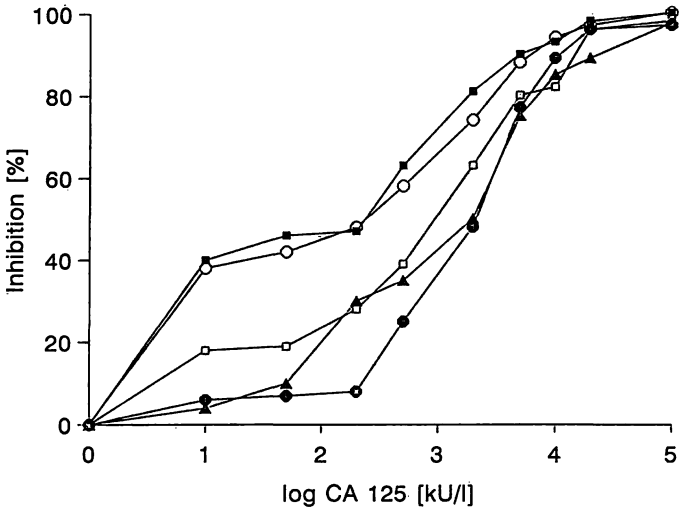

Fig. 5. Inhibition of the binding activity of anti-anti-idiotypic IgG (anti-CA 125) by increasing concentrations of CA 125 antigen (▲—▲ mouse 1, ■—■ mouse 2, ●—● mouse 3, ○—○ mouse 4, □—□ mouse 5).

The peroxidase-labelled goat anti-mouse-IgG (F_c-specific) did not bind to the $F(ab)_2$-fragments of the monoclonal antibody OC125 MAb. Incubation of peroxidase-labelled goat anti-mouse-IgG (F_c-specific) in OC125 MAb-$F(ab')_2$-coated microtitre plates yielded in an absorbance of about 0.030 at 495 nm after substrate addition, when read against similarly treated uncoated microtitre plates.

Discussion

In support of previous hypotheses (1, 5) about the activation of the idiotypic network and immunomodulation by application of monoclonal antibodies, this study demonstrates the induction of a murine anti-OC125 and anti-CA 125 response by immunization with OC125 $F(ab')_2$-fragments.

As previously described for an ovarian cancer patient (9), we demonstrate in this study that repeated immunization with this murine antibody induces the formation of anti-OC125 antibodies in mice. The binding of these antibodies to OC125 $F(ab')_2$-fragments was inhibited by the CA 125 antigen, suggesting

that the responsible IgG are anti-idiotypic antibodies binding either at or near the paratope of the OC125 (Ab2β or γ). After immunization with murine antibodies, humans form anti-isotypic and anti-allotypic antibodies to antigenic determinants common on mouse immunoglobulins, as well as highly specific anti-idiotypic antibodies (13–17). In mice, however, antibodies against idiotypic and allotypic determinants can be expected. However, the fact that binding of anti-OC125 antibodies was completely inhibited by CA 125 indicates that there were no detectable concentrations of anti-allotypic antibodies. Also, anti-idiotypic antibodies, binding to idiotypes which are not inhibited by the antigen, as demonstrated in previous studies (18, 19), obviously are not formed. This is in concordance with our results in humans, where binding of anti-idiotypic antibodies to OC125 was inhibited completely by CA 125 (9). On the other hand, the CA 125 molecule by virtue of its large size (M_r about 200 000) may inhibit binding to many idiotopes by steric hindrance, so that it is difficult to determine whether the Ab2 is Ab2β or γ.

The inhibition curves of the five mouse sera showed certain differences, which might be due to a set of anti-idiotypic antibodies with different affinities.

According to the network hypothesis, anti-idiotypic antibodies of the Ab2β type imitate the original antigen of the Ab1 and can serve as an internal image of the nominal antigen. This internal image possibly activates the idiotypic network by producing anti-anti-idiotypic antibodies (Ab3), which bind to the binding sites of both Ab2β and the original antigen. In the animal model, murine antibodies against the tumour-associated antigen CA 125 were also detected. The binding of these antibodies to immobilized CA 125 could be inhibited by purified soluble CA 125, which suggests that these antibodies bind specifically to the CA 125 antigen. Appearance of these anti-CA 125 antibodies (Ab3) can be interpreted as the formation of anti-idiotypic antibodies to the Ab2β type anti-idiotype which imitates the shape of the epitope on the original antigen CA 125. Detection of Ab3 after immunization with Ab1 gives strong evidence for the existence of an immunological network. Antibodies to CA 125 could not be induced by the CA 125 antigen itself, because the mice did not receive any CA 125 and do not express the antigen. According to the network theory, anti-CA 125 antibodies could be provoked by internal image Ab2 against the anti-CA 125 antibody. The variable regions of these Ab2, expressing an mirror image of the CA 125 antigen, are recognized by the immune system of the host and can induce the formation of possibly regulatory Ab3, which bind to the binding site of the Ab2 and to the original antigen.

The inhibition curves and the inhibitory concentrations of CA 125 were different in the examined sera, indicating that, as for the Ab2, a set of anti-CA 125 antibodies with different affinities was formed.

In contrast, all attempts to demonstrate the existence of circulating Ab3 in serum samples containing Ab2 were without success in tumour patients treated with various monoclonal antibodies (8). Detection of Ab3 against a human antigen is of advantage in a model of a different species. For example, mice, unlike humans, do not express the CA 125 antigen, so that circulating anti-CA 125 antibodies cannot be bound by CA 125-bearing tissues or circulating CA 125.

Presumably, not all Ab2 and Ab3 were detected by the present tests. In an immunometric assay, anti-idiotypic and anti-anti-idiotypic antibodies can be detected if the hypervarible regions are accessible and are not bound to circulating immune complexes in the sera of the treated mice. Only if the immune complexes of antigen, Ab1, Ab2 and Ab3 dissociate in the assay can they then be measured. In incubated mixtures of Ab2, Ab3, OC125 and CA 125, individual equilibria are established between the components, and in cases where the affinity of the Ab3 to immobilized CA 125 is greater than to Ab2, the antigen can displace the Ab2 from the binding to Ab3, so that the Ab3 becomes detectable in an assay.

Concerning the Ab2 detection in sera of immunized mice, only anti-idiotypic antibodies with higher affinity to the OC125 than to the Ab3 are detectable.

Anti-idiotypic antibodies were found in tumour patients after treatment with monoclonal antibodies of different specificities (8, 9, 18, 20, 21). The induction of anti-idiotype Ab2 is correlated with a delayed clinical response in these patients (6, 8, 11, 22).

Our first results in patients with advanced ovarian carcinomas indicate that, in spite of the same surgical and chemotherapeutic treatment, the survival rate is improved after vaccination with antibody-fragments of the OC125 during repeated immunoscintigraphy (10, 11). In these patients showing clinical benefit, the appearance of anti-idiotypic antibodies was demonstrated (9); furthermore, we found an induction of a tumour-specific cellular immunity against autologous tumour tissue, parallel to the production of anti-idiotypic antibodies (23).

The present investigation demonstrates the activation of the idiotypic network from Ab1 to Ab2 and at

least the production of Ab3. The interpretation of the immune system as an idiotypic network provides a basis for inducing an alteration in the immunological response of a tumour patient, which specifically breaks the existing antitumour tolerance, resulting in a beneficial clinical response. Furthermore, this system offers the possibility of immunization against tumour-associated antigens without using the original antigen.

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